REMARKS

Interview Summary

Applicants wish to thank Examiner Woitach for extending the courtesy of the telephonic interviews held on June 8, 2004 and July 7, 2004 with Applicants' representatives Carol Fang and Eugenia Garrett-Wackowski. During this interview, a number of issues were clarified which have helped Applicants to more fully address the concerns of the Examiner. Applicants thank Examiner Woitach for his time.

In particular, during the interviews, the rejection of claims 2-5 under 35 U.S.C. § 112, first paragraph was discussed. Examiner Woitach requested that the Applicants submit additional references describing synchronization of cells at a different stages of the cell cycle using electromagnetic radiation and indicated that submission of such references would fully address the rejection of the claims.

Status of the Claims

After entry of this amendment, Claims 1-5, 7-10, 12 and 46 are pending. Claims 1, 7-10, 12 and 46 have been deemed allowable. Claim 5 has been amended. Support for this amendment can be found throughout the specification, the drawings and the claims as originally filed. Thus, no new matter is introduced by this amendment.

Rejections 35 U.S.C. § 112, First Paragraph

Claims 2-5 are rejected under 35 U.S.C. §112, first paragraph as allegedly lacking enablement.

An invention is deemed to be enabled if one of skill in the art can make and use the invention without undue experimentation. *In re Wands*, 858 F.2d at 737 (Fed. Cir. 1988). As set forth in MPEP § 2164.06, even if the practice of the claimed invention requires a considerable amount of experimentation, it is not necessarily "undue" experimentation:

> The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In*

re Wands, 8 USPQ2d 1400 (Fed. Cir. 1988) (citing *In re Angstadt*, 190 USPQ 214 (CCPA 1976).

Also, a patent does not need to teach that which is well known in the art. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991).

The present invention is directed to methods of increasing the efficiency of transfection of cycling cells sensitive to electromagnetic radiation by synchronizing said cells by contacting said cells with electromagnetic radiation (*i.e.*, Gamma rays, X-rays, or ultraviolet rays);and transfecting the cells within about one cell cycle with a nucleic acid that encodes a desired gene product. The efficiency of transfection is increased over cells not contacted with said electromagnetic radiation.

The Examiner initially alleged that it is well known in the art that radiation (*i.e.*, gamma rays, x-rays, and ultraviolet rays) synchronize mammalian cells at the G2 checkpoint (*see, e.g.*, Office Action, page 3) and concluded that the specification does not enable synchronization of cells at other stages of the cell cycle. As discussed with the Examiner, it was known in the art at the time of filing that electromagnetic radiation could be used to synchronize cells at multiple stages of the cell cycle. In particular, two references that describe synchronization of cells at various stages of the cell cycle were discussed, *i.e.*, Bernhard *et al.*, *Radiat. Environ. Biophys.* 34:79 (1995) and Hwang and Muschel, *Radiation Research* 150(Suppl):S52 (1998) (copies enclosed as Appendices A and B) were described. Both of these references disclose the use of electromagnetic radiation to synchronize cells at various stages of the cell cycle. In particular, Bernhard *et al.* disclose that irradiation of eukaryotic cells can delay progression through the G_1, S, and G_2 phases of the cell cycle or accumulation of cells in S phase. As explained by Bernhard *et al.*, the dose of radiation determines the phase (*see*, page 79, abstract and col. 2, lines 18-35). Hwang and Muschel also disclose that irradiation of eukaryotic cells can delay or arrest cells in the G_1, S, and G_2 phases of the cell cycle. Therefore, as noted by the Examiner, these references demonstrate that of skill in the art would be able to synchronize mammalian cells at many stages of the cell cycle, not just the G_2 phase, with, at most, routine experimentation.

In view of the foregoing, Applicants respectfully submit that the presently claimed invention is enabled by the specification and respectfully request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claim 5 is rejected 5 under 35 U.S.C. § 112, second paragraph as allegedly indefinite for the recitation "the nucleic acid accumulates in the cells." As discussed with the Examiner, claim 5 has been amended for clarity to delete the recitation. Accordingly, Applicants respectfully request withdrawal of this rejection under 35 U.S.C. § 112, second paragraph..

Statutory Type Double Patenting

Claim 5 is rejected under 37 C.F.R. § 1.75 for statutory type double patenting, as allegedly being a substantial duplicate of Claim 4. In making this rejection, the Examiner alleges that the recitation in claim 5 of "the nucleic acid accumulates in the cells" and "have cycled to the G2/M phase boundary" is encompassed by claim 4." As discussed with the Examiner, claim 5 has been amended for clarity to recite "other than a stage selected from the group consisting of: the M phase, late S phase, and the G2/M phase boundary." and to delete the recitation "and the nucleic acid accumulates in cells that have cycled to the G2/M phase boundary." Accordingly, Applicants respectfully request withdrawal of this rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at 415-576-0200.

Respectfully submitted,

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ORIGINAL PAPER

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Effects of ionizing radiation on cell cycle progression

A review

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Abstract Irradiation of normal eukaryotic cells results in delayed progression through the G1, S, and G2 phases of the cell cycle. The G1 arrest is regulated by the p53 tumor suppressor gene product. Irradiation results in increased expression of p53, which in turn induces a 21 kDa protein, WAF1/Cip1, that inhibits cyclin CDK kinases. S-phase delay is observed after relatively high doses of radiation. This delay has both radiosensitive and radioresistant components, corresponding to inhibition of DNA replicon initiation and DNA chain elongation, respectively. The mechanism for this delay is as yet undefined, but the extent of the delay appears to be under genetic control and is sensitive to the kinase inhibitor staurosporine. A delay in G2 has been demonstrated in virtually all eukaryotic cells examined in response to irradiation. Our studies have focused on the mechanisms responsible for this delay. Cyclin B1 and $p34^{cdc2}$ are cell cycle control proteins that together form a kinase complex required for passage through G2 and mitosis [22]. Control of radiation-induced G2 delay is likely therefore to involve modulation of cyclin B1/$p34^{cdc2}$ activity. We have shown in HeLa cells that cyclin B1 expression is decreased in a dose-dependent manner following irradiation. This decrease is controlled at both the level of mRNA and protein accumulation. We have also shown that radiation-sensitive rat embryo fibroblast lines (REF) immortalized with v- or c-*myc* display a minimal G2 delay when compared to radiation resistant cells transformed with v-*myc* + H-*ras*. These REF lines respond to irradiation with a decrease in cyclin B mRNA, which parallels the extent of their respective G2 delays. The duration of the G2 delay in radiation-resistant REF can be shortened by treatment with low doses of the kinase inhibitor staurosporine. We have also been able to markedly reduce the radiation-induced G2 delay in HeLa cells using either staurosporine or caffeine. Attenuation of the G2 delay is accompanied by reversal of the radiation-induced inhibition of cyclin B mRNA accumulation. The results of these studies are consistent with the hypothesis that reduced expression of cyclin B in response to radiation is in part responsible for the G2 delay. The duration of the G2 delay may also be influenced by the activation state of the cyclin B/$p34^{cdc2}$ complex.

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Introduction

Irradiation of a wide range of eukaryotic cells has been shown to induce slowing of cell cycle progression. This lag has several components that can include delays in the G1, S and G2 phases of the cell cycle. Initial studies on radiation-induced cell cycle delays were carried out in HeLa, a human cervical adenocarcinoma cell line. After irradiation, this cell line demonstrated a transient division delay, accompanied by accumulation of cells in S phase [24]. With lower doses of radiation, no S-phase delay was seen, but the duration of the G2 phase of the cell cycle was increased in a dose-dependent manner [33]. The phase of the cell cycle in which cells were irradiated influenced the extent of division delay, with cells irradiated in G1 demonstrating minimal delay, while cells irradiated in S and G2 showed progressively greater delays [28]. Similar findings were obtained in studies using Chinese hamster cells [34] and mouse L-cells [32]. As with HeLa cells, the G2 delay was predominant, but delays in G1 and S were also observed.

Cell cycle arrest in G1 involves p53

The arrest in G1 in response to irradiation is thought to result from a signal transmitted via the p53 tumor suppressor protein in response to cellular damage. The level of p53 protein rises 1–2 h following irradiation and remains high for up to 72 h [11]. This rise is associated with a G1 arrest in cells expressing wild-type, but not mutant p53 protein. In addition, p53 knockout transgenic mice have been usd to demonstrate that G1 arrest in response to radiation requires p53, with the degree of G1 arrest depending on the level of p53 expressed [13]. The ability to arrest in G1 in response to irradiation has also been demonstrated to be predictive of p53 status in human cells [23].

The p53 proteins acts as a transcriptional activator causing increased synthesis of regulatory proteins implicated in cell cycle arrest (Fig. 1). Increased p53 expression, triggered by DNA damage, leads to induction of a 21 kDa protein called Cip 1 or WAF 1 [4]. This protein co-immunoprecipitates with cyclins A, D_1, and E, and has been shown to inhibit phosphorylation of histone H1 by cyclin/cdk2 and cyclin/cdk4 complexes in vitro [7].

Another candidate as a regulator of G1 arrest is the *gadd*45 gene product. *Gadd*45 is one of a series of genes isolated based upon increased expression after DNA damage and growth arrest. Within this series of genes *gadd*45 is unique in that it is strongly induced by DNA damage [25]. Induction of this gene is also dependent upon normal p53 expression. The influence of *gadd*45 on G1 arrest, however, undefined at present.

Characteristics of S phase delay

The delay in progression through S-phase of the cell cycle is due to slowing of the DNA synthesis rate. The dose response of this effect is biphasic, reflecting the presence of both radiosensitive (low dose) and radioresistant (high dose) components in the delay. The radioresistant component is due to a reduction in the rate of DNA chain elongation [31], while the radiosensitive component is due to a decrease in replicon initiation [15].

Studies on the mechanisms of radiation-induced DNA synthesis inhibition comparing radioresistant rat embryo fibroblasts (REF) transformed with H-*ras* + v-*myc* to radiosensitive REF transfected with v-*myc* alone have shown differences in S-phase delay between these cells, indicating the influence of a genetic control mechanism in determining the extent of S-phase delay. Furthermore, the extent and duration of DNA synthesis inhibition in radioresistant REF cells can be attenuated by treatment of these cells with the kinase inhibitor staurosporine, thus implicating a kinase-regulated pathway in the control of S-phase delay [30].

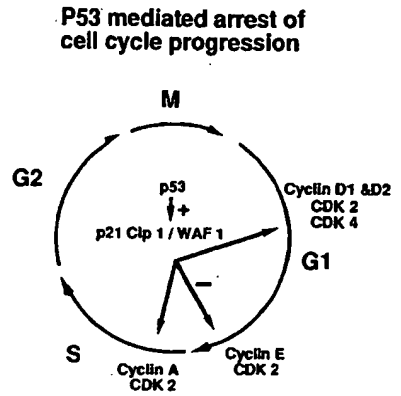

Fig. 1 Diagrammatic representation of potential points in the cell cycle where p53 induced *WAF1/Cip* 1 may inhibit cell-cycle progression in response to DNA damage or growth-arrest signals. Cell-cycle arrest results from inhibition of cyclin/Cdk kinase activity

Radiation resistance is associated with prolonged G2 arrest in rat embryo fibroblast cell lines

The arrest of eukaryotic cells in G2 after irradiation appears to be universal phenomenon which, unlike G1 arrest, is not influenced by p53 gene status. The extent of G2 arrest can be influenced by the expression of other oncogenes. Our studies into the mechanisms of G2 delay after irradiation began with the observation that REF transformed by H-*ras* plus v-*myc* were markedly more resistant to radiation than the parent primary cells or REF immortalized by transfection with v- or c-*myc* [17]. The mean D_o of H-*ras* plus v-*myc* transformed cells and their subclones was approximately 1.9 Gy compared to a mean D_o of 1.0 Gy for the parent and *myc* immortalized lines.

In order to elucidate the mechanism(s) responsible for the increased radiation resistance of H-*ras* plus *myc* transformed REF, we first compared DNA double-strand break induction, and rejoining in a radiation sensitive c-*myc* immortalized cell line, *myc*REC, (D_o=1.1 Gy); and in a radiation-resistant, H-*ras* plus v-*myc* transformed cell, 3.7, (D_o=1.9 Gy) [10]. Breakage and rejoining were measured using pulse field gel electrophoresis of DNA from cells irradiated in the exponential phase of growth. DNA double-strand breaks were quantified by measuring the fraction of the total DNA released into the gel from agarose plugs in which the irradiated cells were embedded. The results demonstrated similar induction of DNA double-strand breaks in both the sensitive and resistant cell lines. Rejoining of DNA double-strand breaks was also found to be similar by this method. Since the induction of double-strand breaks and the kinetics of DNA double-strand break rejoining were similar in *myc*REC and 3.7 cells, neither DNA damage induction nor repair capacity, as measured by this technique, appeared to be responsible for the difference in radiation sensitivity in the REF system.

These results suggested that the observed differences in radiosensitivity in REF cells must be due to other factors

such as differences in cell-cycle progression between radiation-resistant and sensitive cell lines. Further support for this suggestion came from the findings that mammalian cells deficient in G2 arrest, such as lines derived from patients with ataxia-telangectasia, are highly sensitive to ionizing radiation and do not undergo a G2 delay after radiation exposure [21, 35]. Studies using caffeine provided further support for the role of G2 delay in irradiated cell survival. This drug reduces or abolishes the radiation-induced G2 delay and renders cells more sensitive to irradiation [2].

In order to determine whether G2 delay differed in radiation-sensitive and resistant cells, we compared the G2 delay in parental REF, and *myc* immortalized lines to the G2 delay in H-*ras* + v-*myc* transformed REF [16, McKenna unpublished]. The results demonstrated that the G2 delay was significantly greater in the radioresistant H-*ras* + v-*myc* transformed at all doses examined (5–20 Gy). This finding led to the examination of possible mechanisms for the observed differences in G2 delay.

Cell cycle proteins involved in G2/M progression

Recent discoveries have begun to define the biochemical mechanisms controlling cell-cycle progression and yield clues to potential mediators of G2 arrest (reviewed in [22]). Progression through G2 and M is regulated by $p34^{cdc2}$ kinase activity. This kinase activity is tightly regulated through two mechanisms. One is the binding of $p34^{cdc2}$ to cyclins A and B, and the other is the phosphorylation of $p34^{cdc2}$ (Fig. 2). Although $p34^{cdc2}$ protein is present at essentially equal levels throughout the cell cycle in growing cells, its kinase activity only appears in G2/M. Mitotic cyclin levels rise in S and G2. As cyclin levels rise and the G2/M transition nears, they bind with $p34^{cdc2}$. While the binding of mitotic cyclins to $p34^{cdc2}$ is necessary for its activation, it is not sufficient.

The activity of $p34^{cdc2}$ in mammalian cells is also regulated by phosphorylation of threonine 161, tyrosine 15 and threonine 14. The exact timing of the threonine 161 phosphorylation is undetermined, but evidence suggests that it may facilitate cyclin binding [3]. Upon cyclin binding, $p34^{cdc2}$ is also phosphorylated on threonine 14 and tyrosine 15 by regulatory kinase(s). The phosphorylation of tyrosine 15 is mediated by wee-1 kinase, but the kinase responsible for threonine 14 phosphorylation is currently unidentified. These phosphorylations inhibit $p34^{cdc2}$ kinase activity [26]. Final activation of the cyclin B-$p34^{cdc2}$ complex is dependent upon dephosphorylation of both residues, which is mediated by the cdc 25 phosphatase [8].

The two classes of mitotic cyclins called cyclin A and B are both required for completion of the cell cycle. In HeLa cells, the mRNA levels of cyclin B1 rise at late S phase and peak in G2. The rise in mRNA levels appears to be controlled at least in part at the transcriptional level, but may also occur post-transcriptionally. Cyclin B1 associates only with $p34^{cdc2}$. In contrast, cyclin A contributes to the regulation of both S and G2/M. Cyclin A levels rise

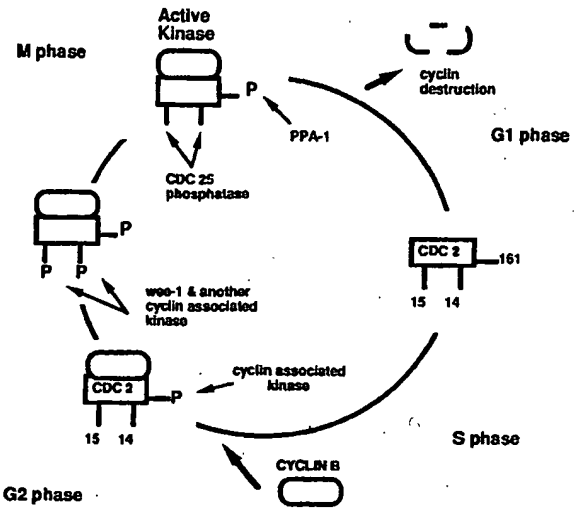

Fig. 2 Illustration of the points in the cell cycle at which $p34^{cdc2}$ associates with cyclin B and undergoes phosphorylation and dephosphorylation. The target sites for phosphorylation are designated numerically corresponding to their position in the protein: threonine at position 14, tyrosine at position 15 and threonine at position 161. The kinases acting on $p34^{cdc2}$ include two as yet uncharacterized cyclin-associated kinases phosphorylating threonines 14 and 161, and a wee-1 like kinase acting at tyrosine 15. Protein phophatase A1 (PPA-1) is thought to dephosphorylate threonine 161. CDC25 activates $p34^{cdc2}$ by dephosphorylating threonines 14 and tyrosine 15

throughout S while cycelin B levels remain low, and it is required for DNA replication [6]. In mammalian cells, cyclin A is found in a complex with a different cyclin-dependent kinase during S phase, $p33^{cdk2}$ [29] and subsequently with $p34^{cdc2}$ during G2 and M. Thus, cyclin A is required for both DNA replication and for the G2/M transition.

The exit from mitosis requires the destruction of both cyclin A and B. If mutations that prevent cyclin destruction are introduced, progression through mitosis into G1 is blocked [12, 18]. Thus, the increase and subsequent destruction of the mitotic cyclins are both required for the cells to transit through G2/M. Because of the pivotal role that the cyclin cdc2 system plays in cell cycle regulation through G2 and M phases, it became the focus of our studies on factors regulating the radiation induced G2 delay.

Radiation induces alterations in cyclin B expression

We initiated our studies into the effects of radiation on the cyclin $p34^{cdc2}$ system by examining the expression of mitotic cyclins after irradiation in HeLa cells [19, 20]. Cells were synchronized at the G1/S border and irradiated with

10 Gy in early S phase. Unirradiated control cells entered G2 phase at 9 to 12 h and had exited from M phase at 15 h. The levels of cyclin B mRNA rose 9 h, peaked at 12 h coincident with the greatest percentage of cells in G2/M phases and fell as the cells re-entered G1 phase. In contrast, irradiated cells did not exit from M phase until after 24 h. Thus, irradiated cells remained in G2 for at least 9 h longer than the unirradiated controls. An S-phase delay was also observed, but the arrest in G2 was predominant. Concurrent with the accumulation of more than 80% of the cells in G2, cyclin B mRNA remained at low levels. Cyclin B protein accumulation was also delayed. In later experiments, we showed that the decrease in cyclin B mRNA and protein was dose-dependent and could be detected at doses as low as 2 to 4 Gy [20]. Thus, after irradiation in S, the accumulation of both cyclin B mRNA and protein were suppressed coincident with the arrest of cells in G2.

Cyclin A expression after irradiation in S was also examined [20]. In contrast to cyclin B, cyclin A mRNA and protein levels rose at the expected time and continued to rise to levels greater than those found in the control cells. After the cells exited from the block and entered G1, the cyclin A mRNA and protein levels fell. These findings are consistent with the hypothesis formulated by Ruderman that cyclin B is responsible for triggering the destruction of cyclin A [14].

These results further indicate that irradiation has some specificity in its effects on both cyclin B mRNA and protein levels since cyclin A expression was not decreased. Although some genes have been found to be induced in cells after irradiation, the expression of many others is unaffected [1, 5, 9, 27]. Thus, the effects on cyclin B mRNA and protein are not a general consequence of irradiation on protein or RNA synthesis and the pattern of their altered expression, while not unique, is unusual.

The effect of irradiation in G2 was also examined [19]. Synchronized HeLa cells were irradiated in G2 phase with 6 Gy of X-rays, at a time when cyclin B mRNA was already increasing. Despite the pre-existing accumulation of cyclin B mRNA, cyclin B protein dropped to very low levels. The levels of cyclin B protein in the irradiated cells did not begin to rise rapidly until after 6 h, corresponding to the exit of the cells from the radiation-induced G2 block. Thus, cyclin B levels are depressed after irradiation in G2, but apparently through a mechanism involving post-transcriptional inhibition of expression.

These data suggest that a cellular mechanism linked to cell-cycle control protein expression recognizes DNA damage and that activation of this mechanism results in decreased cyclin expression. The data further suggest that one target of this mechanism involves redundant controls on the levels of cyclin B protein.

We have also compared the levels of cyclin B expression in radiation-sensitive and radiation-resistant REF transfectants. Synchronized cultures of 3.7 and MR4 were irradiated in early S-phase. The cell cycle transit and accumulation of cyclin B mRNA were then monitored. The radiation-resistant 3.7 REF line exhibits a G2 delay of approximately 10 h after irradiation with 10 Gy. This delay is accompanied, as in HeLa cells with a marked decrease in cyclin B mRNA [Bernhard et al. unpublished data]. In contrast, the radiation-sensitive MR4 cell line (D_o=1.0) exhibited a G2 delay of approximately 2 h. This cell line also failed to show a significant inhibition of cyclin B accumulation. A delay in the decrease of cyclin B mRNA of approximately 2 h was observed corresponding to the short period of G2 delay observed in this cell. These data demonstrate the association between the extent of G2 delay after irradiation and the extent of cyclin B mRNA inhibition.

Conclusions

The continuing elucidation of cell cycle control mechanisms is providing information that is invaluable in uncovering the mechanisms of cell cycle perturbation due to irradiation. The study of cell cycle delay in response to irradiation has similarly contributed to the understanding of cell cycle control mechanisms. The studies described here have established the association between oncogene expression and radiation resistance and further shown that prolonged G2 delay is associated with radiation resistance in REF cells. Decreased cyclin B expression appears to represent one level control over the transit through mitosis after irradiation. Whether other levels of control such as cdc2 phosphorylation alterations are also operative is an area currently being investigated.

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Radiation and the G_2 Phase of the Cell Cycle

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Exposure of mammalian cells to X rays results in prolongation of the cell cycle, including delays or arrests in G_1, S and G_2 phase. While G_1-phase arrest occurs only in cells with wild-type p53 function, a G_2-phase delay occurs in all cells regardless of p53 status. In this review, we summarize what is known about cell cycle progression through G_2 and M phase and discuss the experimental findings that implicate different mechanisms in the G_2-phase delay. Finally, we consider the possibility that G_2-phase arrest plays a role in cell survival after irradiation.

INTRODUCTION

It is a great privilege to write a review about the effects of ionizing radiation on the cell cycle of mammalian cells 100 years after the discovery of radium and the coining of the term radioactivity by Marie and Pierre Curie. That radiation could block cell division has long been appreciated. Alma Howard (another pioneering woman scientist) and Stephen Pelc, in the landmark paper in which they defined the gaps between DNA synthesis and mitosis, G_1 and G_2 phase, noted that irradiation with X rays prolonged both G_1 and G_2 phase in the irradiated cells (*1*). This basic observation was expanded by Yamada and Puck, who observed that irradiation of HeLa cells resulted in a dose-dependent prolongation of G_2 phase (*2*). Terasima and Tolmach further defined the conditions leading to cell cycle alterations by radiation using synchronized HeLa cells to show that a G_2-phase delay occurred regardless of whether cells were irradiated in G_1, S or G_2 phase (*3*). Radiation can have effects on other phases of the cycle. Best understood are the effects on G_2 and G_1 phase, but many experiments have also defined S and M-phase perturbations. A few selected reviews can provide insight into these areas (*4–10*). Our review will concentrate on the G_2 phase of the cell cycle and the alterations in this phase induced by radiation. It will concentrate mainly on the data for mammalian cells and will allude only selectively to experiments with yeast.

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In experiments using irradiated HeLa cells, a G_2-phase block without any G_1-phase block was seen, in contrast to the findings of Howard and Pelc (*1*), who had described both a G_1 and a G_2-phase delay in X-irradiated plant cells. While HeLa cells did not undergo a G_1-phase arrest, many other cell types did. It is now known that expression of wild-type p53 is required for G_1-phase arrest, and many of the tumor cells studied, including HeLa cells, have deficiencies in p53 and hence lack a G_1-phase checkpoint (*11*). The intricacies of this process, however, now suggest that this phenomenon may not be absolute, and the recent work by Li *et al.* indicates that induction of p53 may not always lead to a G_1 arrest, particularly in tumor cells (*12*). Plants have homologues of p53 that probably account for the G_1-phase delay after irradiation first observed by Howard and Pelc, who used plant cells in their studies (*13*). In mammalian cells, p53 exerts much of its inhibitory effect through the transcriptional induction of CDKN1A (p21/WAF1/CIP1), a protein that binds to cyclin–Cdk complexes and hence blocks cell cycle progression at G_1/S phase through inhibition of Cdk1, 4 and 6 activity (*14–18*). While cells deficient in CDKN1A (p21/WAF1/CIP1) have lost much of the G_1-phase delay, a lesser delay remains in some cell types, implying that other unidentified effectors probably also exist (*19, 20*). These cells with defective or absent p53 still undergo a G_2-phase delay after irradiation (*21, 22*).

MECHANISMS REGULATING G_2 PHASE

The G_2-phase delay after irradiation is effected through several mechanisms; this redundancy attests to its physiological importance. In regard to cancer therapy, there is considerable suggestive evidence that the G_2-phase delay can affect survival of cancer cells, and this has raised the possibility that manipulation of this delay might be used to influence response to treatment.

There appear to be multiple redundant mechanisms through which exposure to radiation results in a G_2-phase delay. Very little is known about the signaling triggered by DNA damage that eventually results in the G_2-phase delay. More is known about the downstream mechanisms of cell cycle progression that are affected by radiation. The formation of an active cyclin B1–Cdk1 (or $p34^{Cdk1}$) complex is a central component required for the transition of cells through G_2 phase into mitosis, and considerable work

has focused upon the effects of radiation and other forms of DNA damage upon the activation of this complex. However, there is evidence that p53-mediated activities can affect the G_2-phase delay. Finally, there is the possibility that mechanisms that are independent of both cyclin B1–Cdk1 and p53 have an impact on the G_2-phase delay.

The transition of cells through G_2 phase into mitosis requires the activation of the H1 kinase activity of Cdk1 (*23, 24*). This activity is regulated in two independent ways, first by synthesis of cyclin B1 and second by a series of phosphorylations on Cdk1. Formation of a cyclin B1–Cdk1 complex is required for Cdk1 histone H1 kinase activity. The formation of the complex is triggered by a 10-fold increase in cyclin B1 levels in G_2 phase. Cyclin B1 levels fluctuate through the cell cycle, being low in G_1 phase and highest in G_2 and M phase. Cyclin B1 levels are even lower in G_0 than in G_1 phase. While the increase in cyclin B1 levels in oocytes is dependent entirely on post-transcriptional mechanisms, in somatic cells the levels are regulated by alterations in the levels of the mRNA. Cyclin B1 mRNA levels are controlled both at the transcriptional level and at post-transcriptional levels. The upstream region of the cyclin B1 gene has been cloned and has been shown to have cell cycle-regulated activity throughout the cell cycle and to be inhibited in G_0 phase (*25–27*). In addition to transcriptional control, the stability of the mRNA is also highly variable throughout the cell cycle, with high stability in G_2 and M phase and low stability in G_1 phase (*28*). Thus the increase in cyclin B1 at G_2 and M phase is controlled through increases in mRNA levels that are subject to control both transcriptionally and at the level of mRNA stability. The control has even more facets, since recently we have found that there are two species of cyclin B1 mRNA, one that is cell cycle-dependent and one that is constitutively expressed. The cell cycle-regulated mRNA is translated at a significantly higher efficiency than the constitutive mRNA, suggesting that there may be translation controls on increases in the level of cyclin B1 as well (Hwang *et al.*, unpublished data).

While the presence of cyclin B1 is required for entry into mitosis, its destruction is required for exit from mitosis. This is accomplished by cell cycle-specific ubiquitination that results in a very rapid elimination of cyclin B1 in the final stages of mitosis (*29*). The localization of cyclin B1 may also contribute to its regulation. Cyclin B1 is cytoplasmic for most of the cycle until late G_2 phase, when it is found in the nucleus (*30, 31*). The transport appears to be controlled by phosphorylation of cyclin B1, and this regulation is crucial since many of the targets of the cyclin B1–Cdk1 complex are in the nucleus (*32*).

Thus a variety of mechanisms control the cyclical appearance and localization of cyclin B1 through the normal cell cycle. In addition to the level of cyclin B1, however, the activity of Cdk1 is regulated by a series of phosphorylations. Phosphorylation of the threonine at position 161 of Cdk1 occurs first. This event is catalyzed by CAK (*33–38*).

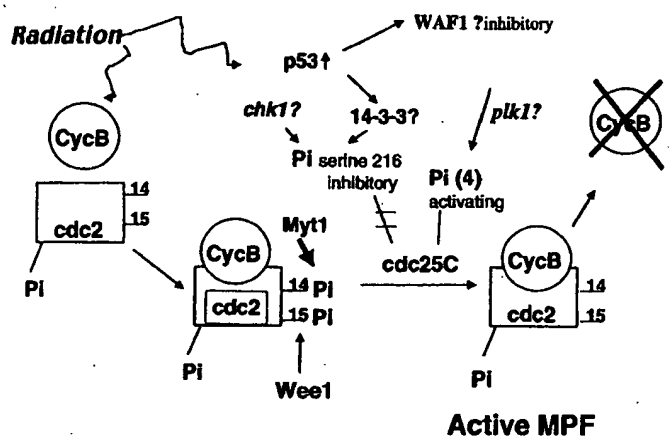

FIG. 1. Depiction of the G_2 phase of the cell cycle. The various reactions affecting the progression of the cell cycle in G_2 phase described in the text are diagrammed here. Pi refers to a phosphate group, and the numbers indicate the amino acid that is affected in the phosphorylation reaction. Cyc B is an abbreviation for cyclin B1. The other molecules indicated are explained in the text.

At the same time that the binding to cyclin B1 occurs, inhibitory phosphorylations at positions threonine 14 and tyrosine 15 (the numbering used here is for the human gene) block the activity of the complex. The phosphorylations on position tyrosine 15 are thought to be mediated by Wee1 kinase and that on threonine 14 by Myt1, a membrane-bound kinase (*39–44*). Enzymatic activity of the complex is generated through the activation of cdc25C, a dual-specificity phosphatase that removes both the phosphates at position 14 and 15 (*45–48*). These biochemical reactions are represented graphically in Fig. 1.

Because of the central role of the cyclin B1–Cdk1 complex and its activity in progression through G_2 into M phase, the effects of radiation on its activity have been a focus of many studies. Lock and Ross first showed that treatment of HeLa cells with X rays or with etoposide led to a G_2-phase block that was accompanied by pronounced inhibition of Cdk1 H1 kinase activity (*49*). This result has been confirmed in a variety of cell lines under a variety of conditions (*49–57*). The next question that emerges is what the mechanisms are that result in this inhibition. The total amounts of Cdk1 protein remain constant through the cell cycle and also after irradiation (*49, 50, 58*). However, the levels of cyclin B1, the necessary activator of Cdk1, are affected by the treatment of cells with ionizing radiation. In addition, phosphorylation of Cdk1 is altered after irradiation, as will be discussed below.

CYCLIN B1 AFTER IRRADIATION

Our laboratory and others have shown that, in cells with a prolonged G_2-phase delay, cyclin B1 levels are reduced in a dose-dependent fashion when compared to the normal levels in G_2 phase (*59–62*). This is not a universal response

of cells to radiation. While the depression of cyclin B1 occurs in HeLa cells under some conditions and in melanoma cells, glioma cells, transformed rat embryo cells and U937 cells, it does not occur in immortalized rat embryo cells, Burkitt's lymphoma cells, some squamous cell carcinoma cells, HeLa cells under some conditions or yeast (*53*, *62–68*). Nonetheless, the depression of cyclin B1 could prolong the length of the G_2-phase delay in tumor cells. To examine this possibility, an inducible expression vector for cyclin B1 was introduced into HeLa cells (*69*). Induction of cyclin B1 did not alter the timing of the normal cell cycle, but after irradiation the induction of cyclin B1 greatly reduced the length of the G_2-phase delay, as would have been predicted if the levels of cyclin B1 help control the length of the G_2-phase delay after irradiation (*69*). Thus in HeLa cells depression of cyclin B1 could be shown to be causal in contributing to the prolongation of G_2 phase. However, this result still leaves open the nature of other mechanisms that can contribute to the production of a G_2-phase delay, which will be discussed below.

The decreased levels of cyclin B1 protein after irradiation are paralleled by decreased levels of mRNA. Maity *et al.* showed that the stability of the cyclin B1 mRNA fluctuates through the cell cycle, but that its stability is reduced almost fivefold after irradiation (*28*). The decreased stability of cyclin B mRNA after irradiation is specific and cannot be generalized to many other messages, although topoisomerase I also is destabilized by radiation (*70*). It should be noted that the expression of some other mRNAs has been shown by Woloschak *et al.* (*71*) to be decreased by radiation. Transcription also regulates the levels of cyclin B1 mRNA, with the highest levels of transcription in G_2 and M phase when the expression of cyclin B1 is highest. Using a promoter linked to a reporter gene, we have found a twofold decrease in transcription due to irradiation, making the destabilization of the message appear to be the major component leading to lowered cyclin B1 levels after irradiation (results not shown).

PHOSPHORYLATION OF Cdk1 AFTER IRRADIATION

Many studies have explored the hypothesis that the inhibition of Cdk1 activity is due to increased inhibitory phosphorylation of Cdk1. Consistent with this possibility is the presence of increased levels of tyrosine phosphorylation on Cdk1 after irradiation (*56*, *57*, *72–76*). However, since phosphorylation of Cdk1 is a normal event during G_2 phase, the question arises whether this is the cause of the G_2-phase arrest or whether it is a consequence of the cell cycle block. It is possible that the accumulation of tyrosine phosphorylations occurs because the cell cycle is blocked at a distal point. These issues have been explored in two ways: first, by attempting to prevent the phosphorylations to determine if this will eliminate the G_2-phase delay, and second, by examining the activity of enzymes known to affect the phosphorylation of Cdk1.

Both Jin *et al.* (*77*) and Blasina *et al.* (*78*) have attempted to ask whether the inhibitory phosphorylations are causal in producing a G_2-phase block. Both groups have employed the strategy of introducing a Cdk1 with mutations at the two positions of inhibitory phosphorylation. The hypothesis was that such a Cdk1 could not be inhibited by phosphorylation, and hence if inhibition of Cdk1 by phosphorylation was the predominant mechanism leading to the G_2-phase delay, the mutant Cdk1 should abrogate the G_2-phase delay. Jin *et al.* found that induction of the mutant form of Cdk1 did reduce the delay, but only a relatively small amount, from 24 to 16 h. In contrast, Blasina *et al.* found that the constitutively active Cdk1 was toxic and that its expression led to cell death, although not through apoptosis. Thus these experiments have not resolved the issue of whether inhibition of Cdk1 occurs via phosphorylation after irradiation.

Many of the effectors of phosphorylation of Cdk1 have also been examined for their potential contribution to the G_2-phase delay. The activity of Wee1, the tyrosine kinase thought to be responsible for the inhibitory phosphorylation on tyrosine 15 in the normal cell cycle, does not appear to be altered after UV or X irradiation (*76*, *79*, *80*). Myt1 is the kinase thought to phosphorylate threonine 15 during the normal cell cycle. Its activity has not been evaluated after irradiation. Cdc25C is the phosphatase that removes both phosphorylations. It too is controlled by phosphorylation. It is phosphorylated on at least four sites of which three have been demonstrated to be activating and one has been implicated as an inhibitory site (*81–84*). The activity of cdc25C has been shown to be reduced after DNA damage by nitrogen mustard and also after irradiation (*74*, *85*, *86*). The loss of activity is coincident with the absence of phosphorylation as revealed by "gel shift" after these treatments. Thus the suggestion has been made that inhibition of activation of cdc25C may lead to persistent phosphorylation of Cdk1, hence blocking cell cycle progression in G_2 phase. Again, the difficulty has been in determining whether the alterations in cdc25C are cause or effect.

While some of the phosphorylations of cdc25C can be mediated by Cdk1, leading Hoffmann *et al.* to hypothesize that activation of cdc25C by phosphorylation is part of a positive feedback loop, activation of cdc25C appears to precede that of Cdk1 and in *Xenopus* oocytes can take place in the absence of Cdk1, suggesting that another kinase may be responsible for this activation in the normal cell cycle (*45*, *81*, *87–90*). One such kinase called plx1 has been identified in *Xenopus* oocytes, although its role in the cell cycle has not been confirmed (*91*). Perhaps to prevent premature activation of cdc25C, multiple phosphorylations of cdc25C are required to induce its phosphatase activity. Izumi and Maller showed that mutations at at least three sites of phosphorylation were required to block activity of cdc25 in *Xenopus* oocytes (*82*), and Strausfeld *et al.* found that cdc25C was active *in vivo* after microinjection only if it had been phosphorylated first (*83*). The phosphorylation sites on cdc25C create a unique epitope called MPM2 that is shared by other phosphorylated proteins in G_2 phase.

Kuang and colleagues have shown that these are not mediated by Cdk1 and have identified an MPM2 kinase (*88–90, 92, 93*). All MPM2 phosphorylations appear to be absent in irradiated G_2-phase cells, indicating that irradiation must be acting at an upstream level in the kinase cascade that leads to cell cycle progression (*86*). This may account for the pleiotropic effects of radiation on the cell cycle machinery.

Recent work examining a potential upstream regulator of cdc25C has also pointed to cdc25C as a target for radiation-induced changes that affect the cell cycle. *Chk1* is a gene that was identified in *Saccharomyces pombe* as being required both for the radiation-induced G_2-phase checkpoint and for the S-phase checkpoint that prevents cell cycle progression before DNA synthesis is complete (*94–96*). Genetic evidence suggests that cdc25C and perhaps also Wee1 are on the pathway through which *Chk1* acts in yeast. Sanchez *et al.* (*97*) identified a human homologue of *Chk1* and determined that it was a kinase that could use cdc25C as a substrate. Peng *et al.* identified serine residue 216 as a target for its action and have suggested that this is an inhibitory phosphorylation (*84*). In their model, Chk1 is activated by DNA damage. It phosphorylates cdc25C, blocking its action and thus preventing cell cycle progression. In their experiments cdc25C phosphorylation at position 216 was difficult to detect, and in the cases in which the inhibitory phosphorylation was observed, the activating phosphorylations also were not seen. These workers have also noted that members of the 14-3-3 family of proteins can bind to cdc25C phosphorylated at serine 216, suggesting that this phosphorylation may lead to inhibition through the binding of this protein. Perhaps phosphorylation of serine 216 leads to binding to 14-3-3 which then prevents the activating phosphorylations from occurring. To determine whether cdc25C was acting in a causal fashion in regulating the G_2-phase delay after irradiation, these workers developed cell lines inducible for either cdc25C or cdc25C with a mutation at serine 216 that abolished phosphorylation. When these proteins were induced, the G_2-phase delay after irradiation was reduced, although it was not eliminated (*84*). This work is the most compelling in suggesting that the phosphorylation of Cdk1 controls the G_2-phase delay and indicates that this could occur through cdc25C. More work is necessary to determine whether the activity of human *CHK1* homologues is stimulated by radiation and how this kinase may affect the radiation response.

There are also data suggesting that the inhibitory phosphorylation of Cdk1 on tyrosine can be mediated by activation of Lyn, a member of the SRC family of kinases. In lymphoma cell lines that express Lyn, Lyn can be found to be associated with Cdk1 and is capable of using Cdk1 as a substrate on its inhibitory sites (*80, 98*).

p53 AND G_2 PHASE

It is likely that the transit through G_2 phase after irradiation is slowed by increased inhibitory phosphorylation on Cdk1, although the evidence remains indirect. Furthermore, the length of the G_2-phase delay in some cell types is regulated by depression of cyclin B1. These conclusions have been reached for the most part based on data for cells without wild-type p53 function. While it is clear that lengthy G_2-phase delays occur after irradiation in cells without p53, demonstrating that p53 is not necessary for the G_2-phase delay, there is also a body of data indicating that p53 itself can effect a G_2-phase delay. Cells that are mutant in p53, that have had a dominant negative form of p53 introduced, or that have homozygous deletions in p53 still undergo a G_2-phase block after irradiation (*10–12*). However, these data do not preclude the possibility that p53 contributes to a G_2-phase delay in a redundant mechanism. Since irradiation or treatment of cells with other toxic agents results in increased levels and activity of p53, its is possible for radiation to effect a G_2-phase delay mediated by p53. Because of the G_1-phase block also induced by p53, experiments to investigate the possibility of a G_2-phase block required special design. When these strategies were employed, induction of expression of p53 in several different cell types was found to induce a G_2-phase delay (*99–101*).

Several mechanisms have been considered in accounting for the ability of p53 to induce a block in G_2 phase. First CDKN1A (p21/WAF1/CIP1) is induced by p53 (*15*). While this inhibitor has been shown to be more active against the G_1-phase cyclin-dependent kinases 2, 4 and 6, it is able to inhibit Cdk1, although with lesser affinity. Thus high levels of p21 in principle could contribute to a G_2-phase block. However, Levedakou *et al.* (*102*) found that the abrogation of induction of CDKN1A (p21/WAF1/CIP1) in murine (12)1KA cells did not alter the G_2-phase delay. Tchou *et al.* (*103*) have suggested that an altered form of CDKN1A (p21/WAF1/CIP1) mediates the G_2-phase delay. Hermeking *et al.* have also identified a potential mediator of the cell cycle block produced by p53 (*104*). Using serial analysis of gene expression (SAGE) to clone transcripts that are differentially expressed, these workers identified mRNAs specifically induced in tumor cells with wild-type p53 after DNA damage. They identified several such transcripts including a member of the 14-3-3 family, 14-3-3 σ, as being induced after irradiation. Coupled with the work of Peng *et al.* (*84*), these studies are beginning to focus attention on the 14-3-3 family as potential regulators of the G_2-phase checkpoint. Kagawa *et al.* have also identified another p53-inducible gene, *GML*, as a contributor to a G_2-phase delay (*105*).

There are also suggestions that mechanisms independent of Cdk1 can lead to a block in G_2 phase. After etoposide treatment, cells arrest in G_2 phase, yet Cdk1 can be active during that arrest. Caffeine also potentiates the induction of Cdk1 H1 kinase activity, yet the cells still remain arrested in G_2 phase (*86*).

CELL SURVIVAL AND THE G_2-PHASE DELAY

The results of the experiments reviewed above lead to the conclusion that the mechanisms leading to the G_2-phase

delay are highly redundant. Since a G_2-phase delay is a universal response by eukaryotic cells to irradiation, it seems likely that cellular integrity depends upon this block, perhaps accounting for the redundancy. Yet the biological functions of the G_2-phase delay remain obscure. It has been posited to be important for survival after DNA damage and has been implicated in maintaining genomic integrity. Supporting the suggestion that the G_2-phase delay may contribute to the survival of cells after irradiation, Hittelman and Rao used premature chromosome condensation during the G_2-phase arrest of mammalian cells to show that many of the DNA breaks were repaired in mammalian cells prior to mitosis (*106*). Weinert and Hartwell isolated a series of mutant cells of *Saccharomyces cerevisiae*, RAD9, RAD17 and RAD24, that failed to arrest in G_2 phase after irradiation (*107*). With RAD9 as the prototype, they showed that this mutation resulted in cells that not only did not delay in G_2 phase after irradiation, but also showed increased sensitivity to cell killing. They thus postulated that the cell cycle contained checkpoints at which cells would arrest in response to DNA damage and that these checkpoints existed to permit cells to repair damage (*108*). There was another class of mutations which resulted in increased radiosensitivity, not because they are defective in cell cycle control, but rather in the machinery that is necessary to effect repair. Cells with mutations in these genes, e.g. *RAD18* and *RAD52*, arrested in G_2 phase because they were unable to repair the DNA damage (*107, 109–111*).

Cell cycle control has been linked to radiosensitivity in mammalian cells as well. As indicated above, irradiated mammalian cells treated with caffeine have decreased survival after irradiation, and caffeine also abrogates the G_2-phase delay (*112–119*). Similar effects can occur with the caffeine analogue pentoxifylline or the kinase inhibitor staurosporine (*120, 121*). Conversely, some cells with a prolonged G_2-phase delay have enhanced radioresistance. We have studied the effects of oncogene transfection on radiosensitivity of rat embryo fibroblasts, and in this system we showed that increased radioresistance was not associated with altered DNA damage or repair, but that the rat embryo cells with a longer G_2-phase delay after irradiation than their counterparts had a significantly enhanced survival after treatment with X rays (*122–124*). A correlation between the length of the G_2-phase delay and radioresistance has also been reported by Su and Little (*125*) after transformation of human cells with SV40. The transformed cells were more resistant to radiation than the parent cells and also had a longer G_2 phase than the parent cells. Jung *et al.* found that induction of the expression of FGF led to increased resistance that was accompanied by a prolonged G_2-phase delay after irradiation (*126*). The results of these studies provided a correlation between the G_2-phase delay and survival but did not provide direct evidence of a linkage.

Finally, the control of G_2 phase may be linked to genetic stability. Cells that are derived from patients with Li-Fraumeni syndrome and that have one mutation in p53 show evidence of increasing genetic instability and aneuploidy with increasing passage. Interestingly, this change in stability correlates with a decreased G_2-phase delay after irradiation (*127, 128*).

In summary, the G_2 phase of the cell cycle is affected by radiation. Regardless of their p53 status, cells undergo a G_2-phase delay. The mechanisms for the delay are only now being dissected. This delay may be mediated by phosphorylation of Cdk1, and its length may be controlled by cyclin B1. It may also be affected by p53 in p53-positive cells. Last, the G_2-phase delay has been suggested to affect survival after irradiation and possibly genetic stability.

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